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Uncovering biosynthetic relationships between antifungal nonadrides and octadrides†

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Maleidrides are a class of bioactive secondary metabolites unique to filamentous fungi, which contain one or more maleic anhydrides fused to a 7-, 8- or 9- membered carbocycle (named heptadrides, octadrides and nonadrides respectively). Herein structural and biosynthetic studies on the antifungal octadride, zopfiellin, and nonadrides scytalidin, deoxyscytalidin and castaneiolide are described. A combination of genome sequencing, bioinformatic analyses, gene disruptions, biotransformations, isotopic feeding studies, NMR and X-ray crystallography revealed that they share a common biosynthetic pathway, diverging only after the nonadride deoxyscytalidin. 5-Hydroxylation of deoxyscytalidin occurs prior to ring contraction in the zopfiellin pathway of *Diffractella curvata*. In *Scytalidium album*, 6-hydroxylation – confirmed as being catalysed by the α -ketoglutarate dependent oxidoreductase ScyL2 – converts deoxyscytalidin to scytalidin, in the final step in the scytalidin pathway. Feeding scytalidin to a zopfiellin PKS knockout strain led to the production of the nonadride castaneiolide and two novel ring-open maleidrides.

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Introduction

Fungal maleidrides are an important family of polyketide-derived secondary metabolites which exhibit a diversity of biological activities including as antifungal^{1,2} and herbicidal^{3,4} agents.⁵ They are characterised by a medium-sized alicyclic ring with one or two fused maleic anhydride moieties. The majority of the reported maleidrides are nonadrides assembled on a 9-membered ring core, and early examples include byssochlamic acid **1**,⁶ heveadride **2**,⁷ glauconic acid **3** and glaucanic acid **4** (Fig. 1).^{8,9} Later studies revealed further nonadrides such as the phomoidrides, e.g. **5**,¹⁰ castaneiolide **6**,¹¹ rubratoxins, e.g. **7**¹² and cornexistin **8**,^{3,13} the octadride viburspiran **9** and more recently two heptadrides agnestadrides A **10** and B have been isolated from *Byssochlamys fulva*.¹⁴ In certain cases the relative

and absolute stereochemistry of the maleidrides remain unknown, and various details of their biosynthesis are yet to be fully elucidated.

Results of extensive studies on selected nonadrides using isotopic labelling, gene knock outs and heterologous expression experiments have led to the proposal that the biosynthetic pathway begins with the assembly of an unsaturated precursor **11**

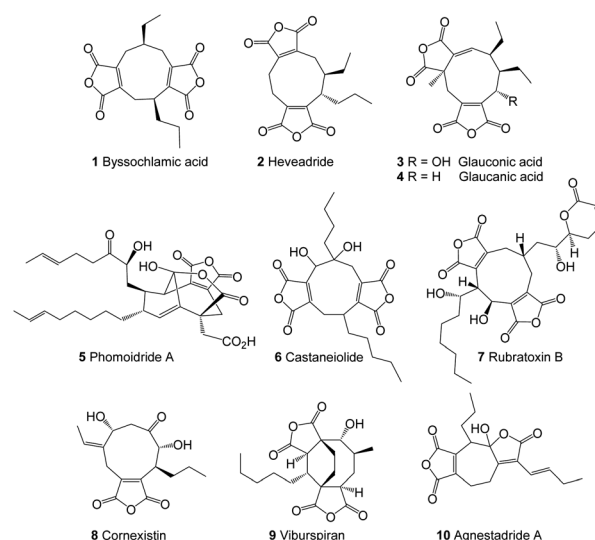


Fig. 1 Examples of maleidrides.

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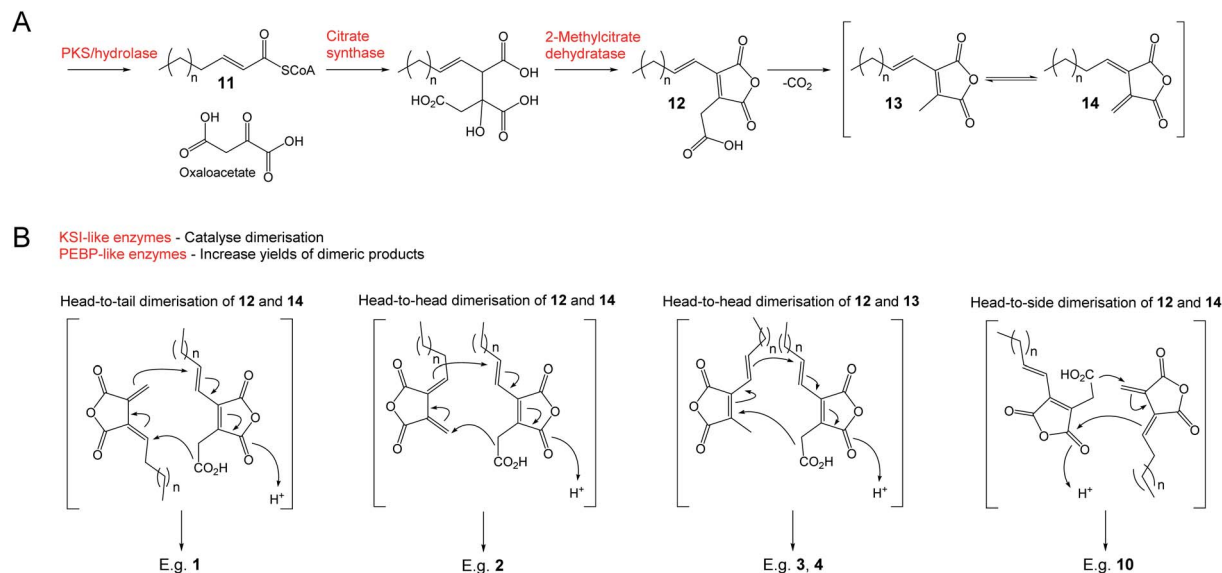
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† Electronic supplementary information (ESI) available. CCDC 2011262–2011264. Crystallographic data for compounds **16**, **17** and **18**. GenBank accession numbers: MT724049 (zopfiellin BGC) and MT724050 (scytalidin BGC). For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/d0sc04309e

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Scheme 1 The universal pathway to maleidrides¹⁶ involves the production of maleic anhydride monomers (A) followed by dimerisation (B) – the mode of which determines the carbon framework of the final compound.

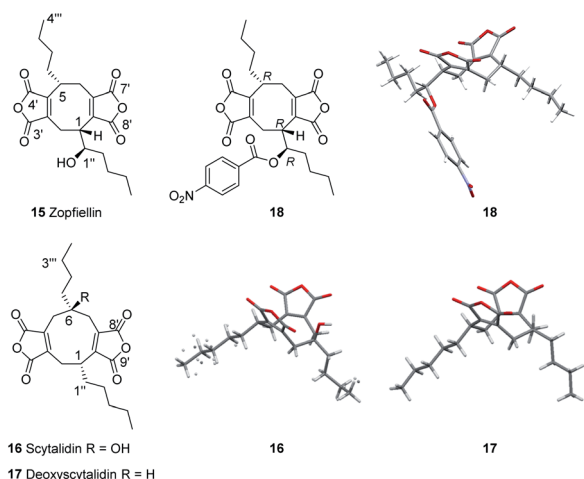


Fig. 2 Confirmed structures of the natural products 15, 16 and 17.

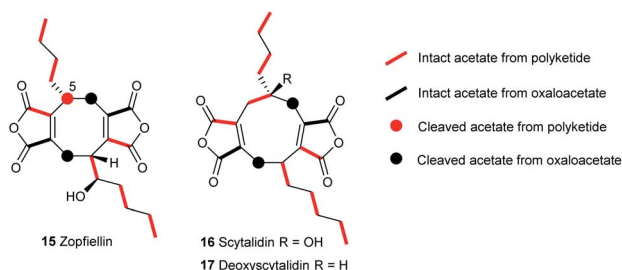


Fig. 3 Incorporation patterns in octadrides and nonadrides from feeding studies with [1,2-¹³C₂]-acetate.

via an iterative highly reducing polyketide synthase (hrPKS), where the chain length varies according to the structure of the natural product (Scheme 1). Coupling of **11** with oxaloacetate is

catalysed by citrate synthase-like enzymes and is followed by dehydration catalysed by 2-methylcitrate dehydratases to generate the maleidride monomer **12**.^{15,16} Decarboxylation of **12** then gives the tautomeric compounds **13** and **14** (Scheme 1A).^{14,17} The second stage of the biosynthetic pathway, coupling the monomers in various modes and cyclisation to the carbocyclic ring, involves ketosteroid isomerase-like (KSI) and phosphatidylethanolamine binding protein-like (PEBP) enzymes.¹⁶ The mode of cyclisation determines the carbon framework of the maleidride (Scheme 1B), and finally tailoring modifications decorate these structures leading to the observed diversity of natural products.

Octadrides, with 8-membered rings, are less common than nonadrides and include viburspiran **9**¹⁸ and zopfiellin **15**¹⁹ (Fig. 1 and 2). The biosynthetic origin of the 8-membered ring is unknown (see footnote). Zopfiellin was first isolated from *Zopfiella curvata* and the structure determined by a combination of spectroscopic methods.^{19–21} The relative and absolute configurations of zopfiellin have been indicated in several publications,^{22–24} but we were unable to find any justification of the configuration of the side chain secondary alcohol. Hence, with our continuing interest in the biosynthesis of fungal natural products, and in particular bioactive maleidrides, our goal was to confirm the structure of zopfiellin **15** and determine the biosynthetic origin of the 8-membered ring, with the longer-term goal of engineering the pathway to new antifungal targets.

Whilst this manuscript was in preparation Minami, Oikawa and co-workers reported investigations into the biosynthesis of zopfiellin using isotopic labelling, heterologous expression of candidate genes in *Aspergillus oryzae*, combined with structure elucidation of minor metabolites. These studies showed that a ring contraction occurs from a nonadride to an octadride.²⁵ Herein we describe our recent studies which confirm the absolute and relative configurations of the nonadride scytalidin **16** and octadride zopfiellin **15** and reveal that they share



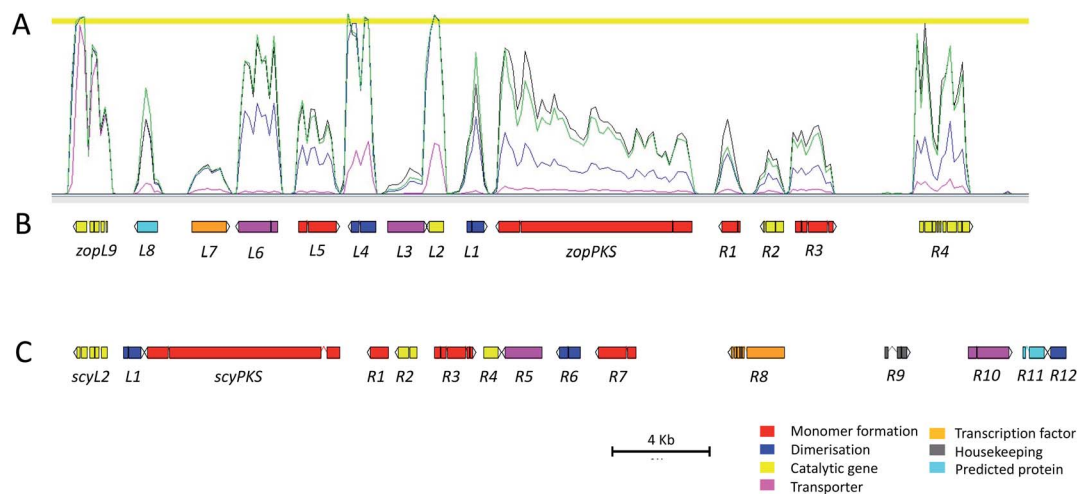


Fig. 4 (A) RNAseq data mapped to the zopfiellin BGC showing coregulation of the genes under four different conditions (Fig. S1 and S2†). Pink: GN. Purple: CDB. Green: PDB-day 5. Black: PDB-day 8. (B) The zopfiellin gene cluster, as annotated in Artemis, which contains genes *zopL9* to *zopR4*. (C) The scytalidin gene cluster, as annotated in Artemis, which contains genes *scyL2* to *scyR12*. 'L' in the gene names denotes genes to the left, or upstream, of the PKS. 'R' denotes genes to the right, or downstream, of the PKS. Putative gene functions are shown in Table 1.

a common biosynthetic precursor, deoxyscytalidin **17** (Fig. 2). Our biosynthetic investigations involved a different approach from those previously reported²⁵ and used a combination of gene disruptions in *DiffRACTella curvata* and *Scytalidium album*, feeding studies to mutant strains and full structure elucidation of known and novel compounds. These studies establish the biosynthetic relationships between zopfiellin **15**, scytalidin **16**, and deoxyscytalidin **17**.

Results and discussion

The confirmed zopfiellin producer, *Zopfiella curvata* no. 37-3, was not publicly available when these studies began, so

DiffRACTella curvata CBS591.74 was obtained and confirmed to produce zopfiellin. Cultures were grown in shake flasks in PDB medium at 25 °C for 14 days and after purification by flash chromatography, gave zopfiellin **15** (80–100 mg L⁻¹) as an oil. As full assignment of NMR data had not previously been reported^{19,21} 2D NMR data (COSY, HSQC and HMBC) analysis allowed assignment of the ¹H and ¹³C NMR spectra (Table S6 and Fig. S31–S36†). The optical rotation, [α]_D –84.3 (c 0.43 MeOH) was consistent with the literature value, [α]_D –76.8 (c 0.42 MeOH).²⁰ Zopfiellin was esterified to give the novel *p*-nitrobenzoate **18** and X-ray crystallography revealed the relative configuration of the natural product (Fig. 2). Synthesis and analysis of the (*R*)- and (*S*)-Mosher's ester derivatives of **15**

Table 1 Predicted functions for genes in the zopfiellin and scytalidin BGCs. Function were determined by searching for homologues within the Swissprot database and identifying conserved protein domains using InterPro.³³ Only the functions of genes marked '*' were assigned via the identification homologues within NCBI's non-redundant database. See Tables S1 and S2 for further details

Gene	Putative function	Homologue	Gene	Putative function	Homologue
<i>zopL9</i>	Hydroxylase/Desaturase	DES ³⁴ AsaB ³⁵	<i>scyL2</i>	Hydroxylase	AcIN ³⁶ Mfr1 ³⁷
<i>zopL8</i>	Hypothetical protein	—	<i>scyL1</i> *	PEBP	BfL5 ¹⁶
<i>zopL7</i>	Transcription factor (TF)	AlnR ³⁸	<i>scyPKS</i>	hrPKS	Tox1-PKS ³⁹
<i>zopL6</i>	Major facilitator superfamily (MFS) transporter	Itp1 ⁴⁰	<i>scyR1</i>	DUF341 hydrolase/esterase	Fub4 ⁴¹
<i>zopL5</i>	2-Methylcitrate dehydratase (2MCDH)	PrpD ⁴²	<i>scyR2</i>	Enoyl CoA hydratase	Ech1 ⁴³
<i>zopL4</i> *	Ketosteroid isomerase-like protein (KSI)	BfL6 ¹⁶	<i>scyR3</i>	Citrate synthase	Mfr3 ⁴⁴
<i>zopL3</i>	Major facilitator superfamily (MFS) transporter	Itp1 ⁴⁰	<i>scyR4</i>	Isochorismatase-like hydrolase/ amidohydrolase	CSHase ⁴⁵
<i>zopL2</i>	Isochorismatase-like hydrolase/amidohydrolase	NicF ⁴⁶	<i>scyR5</i>	MFS transporter	MfM6 ⁴⁴
<i>zopL1</i>	Phosphatidylethanolamine-binding protein (PEBP)	Tf11 ⁴⁷	<i>scyR6</i> *	KSI	BfL6 ¹⁶
<i>zopPKS</i>	hrPKS	Tox1-PKS ³⁹	<i>scyR7</i>	2-Methylcitrate dehydratase	PrpD ⁴²
<i>zopR1</i>	DUF341 hydrolase/esterase	Fub4 ⁴¹	<i>scyR8</i>	Transcription factor	AlnR ³⁸
<i>zopR2</i>	Enoyl-CoA hydratase	Ech1 ⁴³	<i>scyR9</i>	Histone H2A	H2A.Z ⁴⁸
<i>zopR3</i>	Citrate synthase	CshA ⁴⁹	<i>scyR10</i>	MFS transporter	Mfr5 ⁴⁴
<i>zopR4</i>	FAD-dependent oxidoreductase	YanF ⁵⁰ Sol5 ^{51,52}	<i>scyR11</i>	Hypothetical protein	—
			<i>scyR12</i> *	PEBP	BfL9 ¹⁶



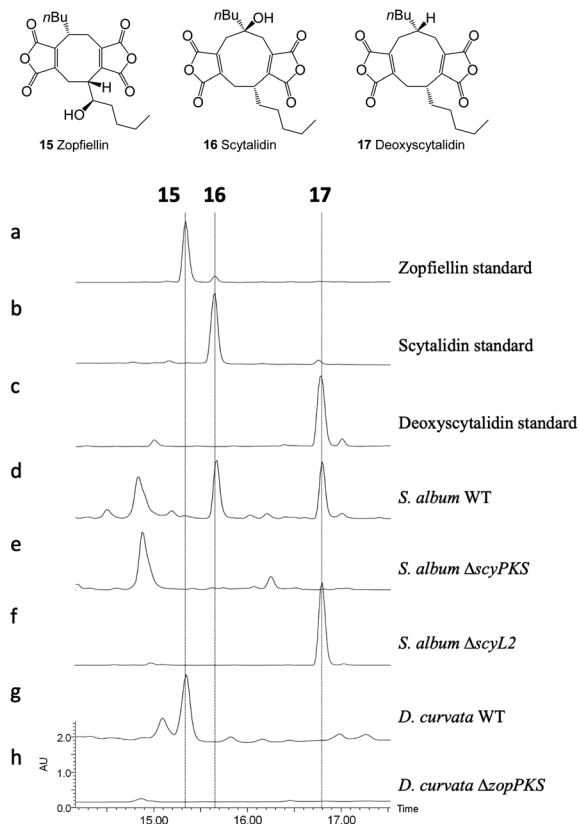


Fig. 5 HPLC (DAD) analysis of gene deletion strains for the zopfiellin and scytalidin gene clusters. Disruption of *zopPKS* and *scyPKS* abolished maleidride production in both species, confirming the identity of the zopfiellin and scytalidin BGCs. Disruption of *scyL2* confirmed its role in the final hydroxylation of the scytalidin pathway.

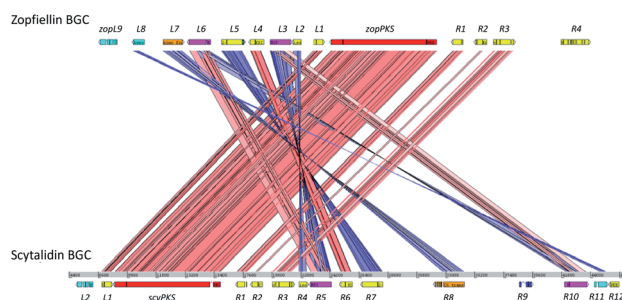


Fig. 6 An ACT (Artemis Comparison Tool) comparison of the zopfiellin and scytalidin BGCs identified genes predicted to encode homologous proteins and highlighted the similarity between the two biosynthetic gene clusters.

confirmed the absolute stereochemistry of zopfiellin as 1*R*, 5*R*, 1''*R* (Fig. S81–S83†).

Feeding [1,2-¹³C₂]-acetate to cultures of *D. curvata* showed mainly intact incorporation of acetate into zopfiellin 15 (Fig. 3). Enhancement of C-5 in the ¹³C NMR spectrum was observed, but there was no coupling to an adjacent ¹³C, indicating that it originates from a cleaved acetate and therefore that a carbon atom is lost during zopfiellin biosynthesis.

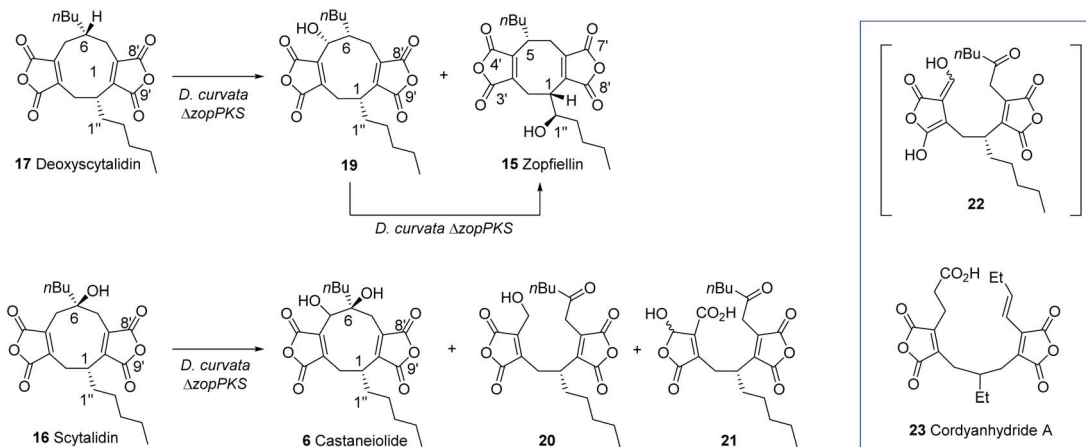
A possible biosynthetic precursor to zopfiellin is therefore a nonadride which on rearrangement with the loss of one carbon atom would give the corresponding octadride. Such ring contractions are well preceded in fungal biosynthesis, for example, in the contraction of ring B of the kaurenoids to the gibberellins,²⁶ the conversion of the 14-membered polyketide macrodiolide colletotriene to the 13-membered ring of bartanol²⁷ and the oxidative ring contractions seen in xenovulene biosynthesis.²⁸ Conceivably the known natural products scytalidin 16²⁹ (C₂₂H₂₈O₇), deoxyscytalidin 17³⁰ (C₂₂H₂₈O₆) or castaneiolide 6¹¹ (C₂₂H₂₈O₈) could be precursors. To obtain samples of 16 and 17, cultures of *S. album* strain UAMH 3620 were grown, and after 14 days compounds 16 and 17 were isolated in similar titres (*ca.* 10 mg L⁻¹). X-ray analysis confirmed that the alkyl side chains are *syn* in both metabolites (Fig. 2). The results of feeding studies using [1,2-¹³C₂] acetate to cultures of *S. album* were in accord with the predicted biosynthetic pathway to nonadrides and that one or both could possibly be biosynthetic precursors of zopfiellin 15 (Fig. 3).

De novo genome sequencing was performed for *D. curvata* CBS591.74 and *S. album* UAMH3620, and an initial screen of the genome data, searching for homologues to the byssochlamic acid biosynthetic gene cluster (BGC),¹⁶ revealed putative maleidride gene clusters within both genomes. The gene clusters were analysed in detail using FGenESh³¹ to predict coding sequences, and manually annotated in Artemis³² to produce full draft BGCs (Fig. 4 and Table 1). Putative gene functions were assigned *via* the identification of homologues in the NCBI database and the detection of conserved domains using InterPro³³ (Tables S1 and S2†). Transcriptomic analysis for *D. curvata* under a range of zopfiellin 15 production and non-production conditions aided identification of the cluster boundaries by clearly identifying a co-transcribed region that includes genes from *zopL9* to *zopR4* (Fig. 4, S1 and S2†). In addition, detailed analysis of the transcriptomic data confirmed the positions of all introns and exons in the predicted ORFs. However, despite repeated attempts, it was not possible to generate quality RNAseq data for the putative scytalidin gene cluster in *S. album*.

To confirm the identity of the two gene clusters, protoplast-mediated transformation protocols were developed for both fungal species and the PKS genes; *zopPKS* and *scyPKS*, were disrupted using the bipartite gene knock-out approach developed by Nielsen *et al.*⁵³ In both cases this led to a total loss of maleidride biosynthesis, with no scytalidin 16 or deoxyscytalidin 17 being present in the crude extracts of the *ΔscyPKS* strains (Fig. 5e), and no zopfiellin 15 being present in the crude extracts of the *ΔzopPKS* strains (Fig. 5h).

An ACT (Artemis Comparison Tool)⁵⁴ comparison of the zopfiellin and scytalidin BGCs (Fig. 6) highlighted significant homologies. Both clusters contain genes encoding the essential hrPKS, DUF341 hydrolase, citrate synthase (CS) and 2-methylcitrate dehydratase (2MCDH),^{12,13,15,16} as well as the 'dimerisation' genes encoding a ketosteroid-isomerase-like protein (KSI) and a PEBP-like protein.¹⁶ In the case of the scytalidin BGC a second PEBP gene is present, which has been observed previously in the byssochlamic acid and rubratoxin BGCs.^{12,16} A domain analysis of the two PKS genes identified all of the





Scheme 2 Feeding studies to *D. curvata* strain $\Delta zopPKS$ identified 17 and 19 as intermediates in zopfiellin biosynthesis and scytalidin was metabolised to novel products.

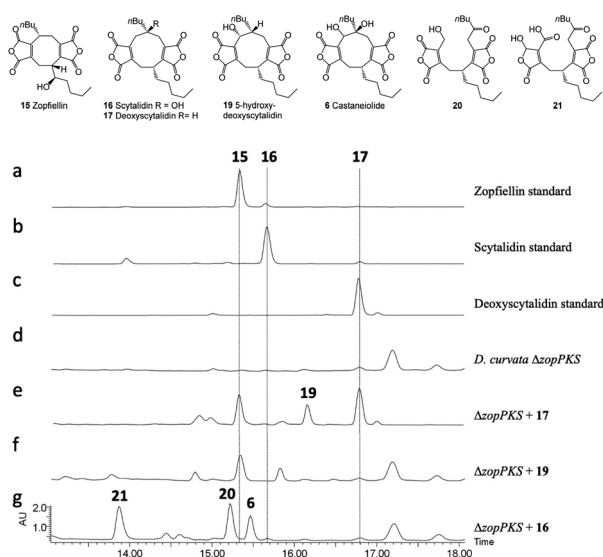


Fig. 7 HPLC (DAD) chromatograms of crude extracts. Feeding compounds to *D. curvata* $\Delta zopPKS$ indicate that deoxyscatalidin 17 and 5-hydroxy-deoxyscatalidin 19 are intermediates in the zopfiellin biosynthetic pathway, whereas scytalidin 16 is not. Feeding of 16 led to the production of castaneiolide 6 and two novel ring-open anhydrides 20 and 21.

domains typically present in a highly-reducing PKS: KS, AT, DH, CMET, ER, KR and ACP. Additional genes identified in both clusters encode an enoyl-CoA hydratase, an isochorismatase, a small hypothetical protein (*zopL8* and *scyR11*), a GAL4 type transcription factor and two MFS (major facilitator superfamily) transporters. The enoyl-CoA hydratase encoded by the zopfiellin cluster (*zopR2*) was not identified in the work by Oikawa and colleagues,²⁵ but RNAseq data confirmed its annotation.

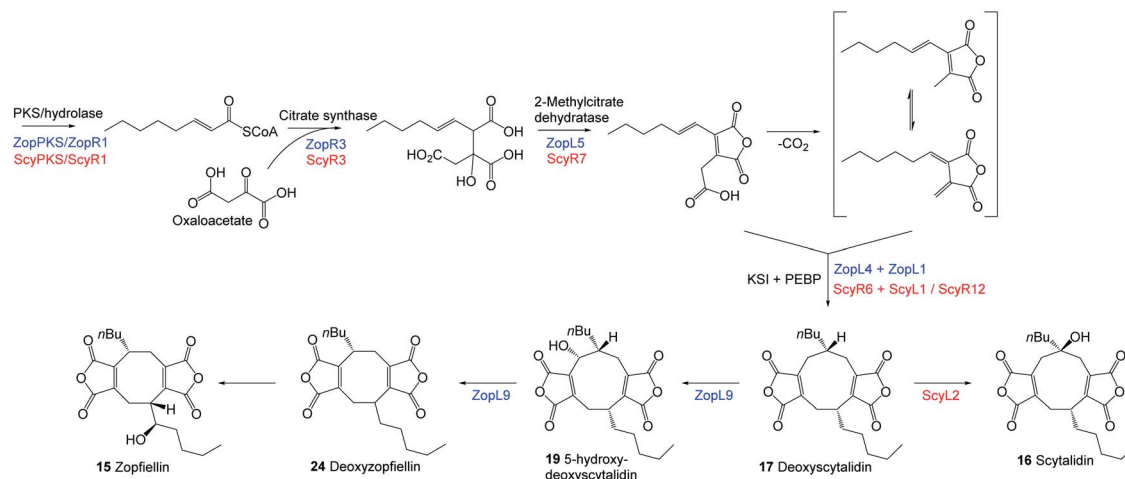
The proteins encoded by the clusters also have high sequence identity (Table S3†). The KSI and PEBP-like proteins, for example, are far more similar to one another (80.7% and 66.2% identity respectively, Table S3†) than to those identified in other maleidride BGCs (Fig. S4–S7†). In the case of the

transporters, ZopL6 and ScyR5 are highly homologous (80.4% identity) and thus may be expected to transport similar compounds. The other two transporters, ZopL3 and ScyR10, are more diverse, sharing only 38.5% identity. All other homologues encoded by the gene clusters share over 50% identity, with many sharing over 80% (Table S3†).

The only proteins which are uniquely encoded by the zopfiellin gene cluster are a putative hydroxylase/desaturase (ZopL9), which shares homology with DES from the gibberellin pathway, and an FAD-dependent oxidoreductase (ZopR4). The scytalidin gene cluster encodes only one unique protein, a putative hydroxylase (ScyL2). ZopL9 and ScyL2 are both predicted to be α -ketoglutarate (α KG) dependent enzymes based on the presence of conserved iron and α KG binding residues (Fig. S10†), but they were not considered to be homologues due to the low level of sequence identity (approximately 25% identity), suggesting that the exact catalytic function is unlikely to be the same. Also located within the scytalidin BGC is a gene (*scyR9*) encoding a highly conserved H2A.F/Z family histone,⁵⁵ but this is unlikely to play a direct role in scytalidin biosynthesis (Table S3†).

The striking similarity between the two gene clusters (Fig. 6) suggests that zopfiellin 15 and scytalidin 16 have very similar biosynthetic origins, and that either deoxyscatalidin 17 or scytalidin 16 may be precursors of zopfiellin. To test this theory, 16 and 17 were purified from *S. album* cultures and separately fed to *D. curvata* $\Delta zopPKS$ cultures on days 3 and 5 post-inoculation (5 mg per day per 100 ml of culture). In cultures fed with 17, zopfiellin production was restored (Scheme 2 and Fig. 7e), demonstrating that deoxyscatalidin 17 is a precursor of zopfiellin. The extent of conversion of deoxyscatalidin to zopfiellin varied between individual feeds, presumably due to varying expression levels for the zopfiellin BGC. In cultures where the conversion was partial, the HPLC trace showed a peak for deoxyscatalidin, as well as a novel compound with a mass of 404 eluting at 16.1 minutes (Fig. 7e). This metabolite was isolated, and the structure confirmed by NMR spectroscopy to be 5-hydroxy-deoxyscatalidin 19 (Table S9 and Fig. S44–S49†). The





Scheme 3 Proposed biosynthetic pathways of the maleidrides deoxyscyltidin 17, scyltidin 16 and zopfiellin 15.

signal assigned to 5-H (at δ 4.90) appeared as a broad singlet and NOE studies were in accord with hydroxylation occurring on the same face as the butyl side chain at C-6. To determine whether 19 is a shunt product or an intermediate on the biosynthetic pathway, it was fed to *D. curvata* Δ zopPKS cultures and again this restored zopfiellin biosynthesis (Scheme 2 and Fig. 7f).

When scyltidin 16 was fed to cultures of *D. curvata* Δ zopPKS, no zopfiellin was detected, but several additional compounds were present when compared to controls (Fig. 7g). The mixture was purified by HPLC giving the known¹¹ nonadride castaneolide 6 as one of the metabolites, presumably formed by 5-hydroxylation of scyltidin 16 (Scheme 2). In addition, two novel compounds 20 and 21 were isolated (3.2 mg and 0.7 mg respectively) and their structures elucidated by MS and extensive NMR studies (Table S10 and Fig. S50–S63†). The ¹³C NMR spectrum of compound 20 (C₂₂H₂₈O₈ from HRMS) showed downfield signals assigned to the two maleic anhydrides, as well as an additional signal at δ_C 205 ppm assigned to a ketone. The ¹H-NMR showed characteristic AB signals assigned to the hydroxymethylene. Based on this evidence, and combined with further analysis of 2D NMR data, the new metabolite was assigned as the ring cleaved alcohol 20. The spectral data of 21 was also in accord with a ring cleaved metabolite with a ketone in the side chain, but in this case the primary alcohol was oxidised to a carboxylic acid and one of the carbonyls of the anhydride reduced to give a mixture of epimers (δ_C 96.9/97.4 ppm, δ_H 6.19/6.23 ppm). Such reductions of anhydrides are commonly observed in maleidride biosynthesis.^{14,56,57} We propose that ketone 20 may be formed *via* cleavage of the 5,6-diol 6 to give an intermediate 22, which would tautomerise to generate ketone 20. Oxidation of the primary alcohol of 20 to a carboxylic acid and selective reduction of the anhydride would give 21. Such linear bis(maleic anhydride) structures are not without precedent, for example the fungal metabolite cordyanhydride A 23.⁵⁸ However, 23 has previously been proposed to arise *via* the linear coupling of two distinct maleic anhydride monomers rather than *via* cleavage of a cyclic intermediate.¹⁵

Based on the bioinformatic analysis of the BGCs and the identification of deoxyscyltidin 17 as an intermediate to both scyltidin 16 and zopfiellin 15, it is a reasonable inference that the two biosynthetic pathways proceed in an identical manner to 17, after which they diverge. Hydroxylation at C-6 would produce scyltidin whereas hydroxylation at C-5, ring contraction and hydroxylation of the side chain would produce zopfiellin.

ScyL2, being the only unique catalytic protein encoded by the scyltidin BGC, was identified as a likely candidate for catalysing the final step in the scyltidin biosynthetic pathway. This is supported by a bioinformatic analysis identifying ScyL2 as a likely α -ketoglutarate dependent hydroxylase (Table S2†), which shares homology with enzymes known to catalyse hydroxylations, such as Mfr1 and Mfr2 from the squalestatin pathway,³⁷ and RbtB and RbtG from the rubratoxin pathway¹² (Fig. S8–S10†). To confirm this role, *scyL2* knock-out strains of *S. album* were generated. These strains did not produce scyltidin 16, but accumulated deoxyscyltidin 17 (Fig. 5f), confirming the role of ScyL2.

Recent work by Shiina *et al.*²⁵ on the zopfiellin pathway of *Z. curvata*, using heterologous expression as well as *in vitro* assays, has begun to elucidate the later stages of zopfiellin biosynthesis. ZopK, which is equivalent to ZopL9 of the *D. curvata* BGC reported here, was shown to catalyse the oxidative ring contraction in the zopfiellin pathway to produce deoxyzopfiellin 24.²⁵ This proceeds *via* the intermediate 19 (which we now know to be 5-hydroxy-deoxyscyltidin) and is consistent with our identification, *via* feeding studies, of 19 as an intermediate in the zopfiellin pathway. *In vitro* assays using purified recombinant ZopL9 gave results which are entirely consistent with the findings of Shiina *et al.*²⁵ In the presence of α -ketoglutarate, ZopL9 was capable of catalysing highly efficient hydroxylation of deoxyscyltidin 17 to give 5-hydroxy-deoxyscyltidin 19 (Fig. S68†). A minor compound was also detected which was purified and identified as deoxyzopfiellin 24 (Fig. S68†), in accord with the



proposal that ZopL9 is implicated in the ring contraction in the zopfiellin pathway of *D. curvata*, by first hydroxylating deoxyscytalidin to give **19**, then catalysing the ring contraction to give the octadride core.

Having identified **17**, **19** and **24** as intermediates in the zopfiellin pathway – through both feeding studies and *in vitro* assays – an extract of wild-type *D. curvata* was re-analysed, which revealed the presence of all three compounds as minor metabolites (Fig. S25†). Taken together with the bioinformatic analysis and the discovery that ScyL2 catalyses the final step of the scytalidin pathway, a branching biosynthetic route for the production of deoxyscytalidin, scytalidin and zopfiellin can now be proposed as shown in Scheme 3.

Intriguingly there is a homologue to ZopL9 (PhiK) encoded by the phomoidride BGC (44.2% homology – Fig. S11†). The structure of the phomoidrides, *e.g.* **5**, suggests that multiple oxidations must occur during their biosynthesis, but no ring contraction of the nine-membered core occurs in this pathway, and the role of PhiK has not yet been investigated.

Conclusions

This work categorically establishes the biosynthetic relationship between the nonadride scytalidin **16** and the octadride zopfiellin **15**, *via* the shared nonadride intermediate; deoxyscytalidin **17**. In both pathways, deoxyscytalidin is the substrate for α KG-dependent dioxygenase enzymes. In the scytalidin pathway ScyL2 catalyses a 6-hydroxylation of deoxyscytalidin to produce scytalidin whereas in the zopfiellin pathway ZopL9 catalyses a 5-hydroxylation of deoxyscytalidin and is implicated in a ring contraction to produce the octadride core. Interestingly, when cultures of *D. curvata* Δ zopPKS were fed with scytalidin, (possessing a 6-hydroxy group), 5-hydroxylation occurred to give castaneiolide **6** and two novel ring-cleaved maleidrides **20** and **21** were produced. Genes encoding α KG-dependent enzymes have been identified within a further three maleidride BGCs; namely *rbtB*, *rbtE*, *rbtG* and *rbtU* of the rubratoxin cluster, and the currently uncharacterised *phiK* of the phomoidride BGC and *pvL5* of the cornexistin BGC. This highlights the important role these, often multifunctional, enzymes play in generating the structural diversity seen in the maleidride class of natural products. Intriguing questions which remain to be answered regarding the biosynthesis of zopfiellin include the mechanism by which the ring contraction occurs, and the identification of the enzyme responsible for installing the hydroxyl group at C-1'' of the side chain of zopfiellin. ZopR4, which is uniquely encoded by the zopfiellin BGC and predicted to be a FAD-dependent oxidoreductase, is a candidate for catalysing such a transformation, but this remains to be explored experimentally. Additionally, the roles of the isochorismatase-like enzyme (ZopL2/ScyR4), the enoyl CoA hydratase (ZopR2/ScyR2) and the small hypothetical protein (ZopL8/ScyR11), which are encoded by both BGCs, are currently unknown.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

§ The numbering systems used for the maleidrides varies greatly in the literature and shows no consistency. We propose a common more systematic system based on the size of the ring (1–9, 1–8, 1–7 as appropriate) beginning at the carbon alpha to the maleic anhydride ring, which gives the lowest numbers to the side chains. The maleic anhydride carbons would be numbered with a prime, appropriate to the ring numbering, hence 3'4' and 8'9' for byssochlamic acid, and 1'', 2'', *etc.* for the first side chain, numbering from the ring junction and 1''', 2''', *etc.* for the second side chain.

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